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GENETIC EVIDENCE OF HYBRIDIZATION BETWEEN *OENOTHERA WOLFII* (WOLF'S EVENING PRIMROSE) AND *O. GLAZIOVIANA*, A GARDEN ESCAPE

Jennifer DeWoody*[1], Leonel Arguello[2], David Imper[3], Robert D. Westfall[4] and Valerie D. Hipkins[1]

[1] USDA Forest Service, Pacific Southwest Research Station, National Forest Genetics Lab, 2480 Carson Road, Placerville, CA 95667

[2] U.S. Department of the Interior, National Park Service, Redwood National and State Parks, P.O. Box 7, Orick, CA 95555

[3] U.S. Fish and Wildlife Service, 1655 Heindon Road, Arcata, CA 95521

[4] USDA Forest Service, Pacific Southwest Research Station, P.O. Box 245, Berkeley, CA 94701-0245

Abstract

Isozyme analysis of the rare *Oenothera wolfii* (Wolf's evening primrose) and the garden escape, *O. glazioviana*, indicates that hybridization between these species may be more widespread than morphological evidence indicates. Although both species contained low amounts of genetic variation, unique alleles were identified in both taxa. Analysis of 22 populations, including pure populations of each species, identified eight populations as containing putative hybrid individuals. Four of these putative hybrid populations were considered pure *O. wolfii* based on morphological analysis. This study confirms that the native *O. wolfii* may be at risk not only from habitat destruction, but potentially from genetic swamping where it co-occurs with *O. glazioviana*. These results can be used as baseline information for future genetic monitoring efforts.

Key Words: complex heterozygote, genetic swamping, hybridization, isozymes, *Oenothera*.

Although habitat loss usually poses the greatest threat to a rare species' survival, there is evidence that hybridization with widespread related taxa poses an immediate threat to some species (Rhymer and Simberloff 1996). A rare species may become functionally extinct through genetic swamping after repeated hybridization and backcrossing with a more common species (Levin et al. 1996). Management efforts to minimize hybridization in order to protect a rare species may not be justified if hybridization results from natural processes. By contrast, artificial hybrid zones arising from human-mediated habitat modification or species introduction may require management action to minimize the potential loss of a rare species (Rhymer and Simberloff 1996; Allendorf et al. 2001). In order to minimize the effects of artificial hybrid zones, managers must be able to distinguish between pure populations of a rare species and hybrid swarms where the two species coexist. Frequently, hybrids display phenotypes intermediate to either parent species, although hybrid morphology may be extreme to either parent (Schwarzback et al. 2001). Due to these variations, morphology alone may be insufficient to completely describe hybrid swarms of individuals, particularly if second-generation hybrids or backcross individuals occur frequently. Genetic information may provide greater power to identify hybrids if unique alleles occur in either or both pure species. Even in the absence of unique alleles, given sufficient variation in neutral, bi-parentally inherited, genetic markers (for example, isozymes), statistical methods exist to identify not only first-generation hybrid individuals, but also second-generation hybrids and introgressed individuals resulting from backcrosses with either parental species (Rannala and Mountain 1997; Rieseberg et al. 1998; Nason et al. 2002).

Oenothera wolfii [Munz] Raven, W. Dietr. Stubbe (Onagraceae) (Wolf's evening primrose) is a biennial to short-lived perennial native to the coastal areas of northern California and southern Oregon. Populations of this species are rare and patchy in distribution, found on moderately disturbed sites, including the upper margin of beach strand and coastal bluffs (Imper 1997). While disturbance resulting from continued development and recreation along the coast have created new habitat for *O. wolfii* in some instances, the net effect of human encroachment has been negative for existing populations (Imper 1997). As a result, *O. wolfii* is listed as threatened by the state of Oregon, and both the California Native Plant Society and the Oregon Natural Heritage Program list this species as endangered throughout its range (Imper 1997).

* Author for correspondence. Current address: University of Southampton, School of Biological Sciences, Southampton, SO16 7PX, United Kingdom. +44-(0)79-8363-0570 (voice), +44-23-8059-4459 (fax), j.dewoody@soton.ac.uk.

While habitat loss is affecting *O. wolfii*, hybridization with a common congener, *O. glazioviana* Micheli, may prove the more immediate threat (Imper 1997). As a garden escape (i.e., a horticultural species of hybrid origin that has become established in natural areas), *O. glazioviana* may be interfertile with *O. wolfii*, producing artificial hybrid zones where the species coexist. Several factors support this hypothesis. First, introgression is common between many members of this genus. Greenhouse experiments have shown that hybridization between *O. wolfii* and other members of the genus readily occurs (Wasmund and Stubbe 1986). Second, individuals of hybrid origin have been identified at the California-Oregon border area based on morphological traits (Carlson et al. 2001). Hybrids are fertile, vigorous, and display a greater fitness than either parent species (Imper 1997). Although genetic typing of hybrid individuals indicates that hybrids tend to breed true, there is evidence of hybrids back-crossing with *O. wolfii* (Imper 1997). Third, *O. wolfii* is potentially susceptible to genetic swamping by *O. glazioviana* based on the mating systems of each species. *O. wolfii* is self-compatible and produces the majority of its seed via self-pollination (Carlson et al. 2001). This breeding system is a consequence of *O. wolfii*'s complex heterozygous genome, which is maintained through self-fertilization and balanced lethals, and results in approximately half of the mature pollen grains being sterile (Wasmund and Stubbe 1986). In contrast, *O. glazioviana* is an outcrossing species (Imper 1997). Given the asymmetry of available pollen between these parent species asymmetric gene flow might occur as *O. glazioviana* pollen swamps *O. wolfii* stigmas at sympatric sites. Together, this evidence suggests that hybridization likely occurs between this rare endemic and the widespread garden escape.

This study reports an investigation into the extent and structure of hybrid zones between *O. wolfii* and *O. glazioviana* using isozymes, which are putatively neutral, bi-parentally inherited, molecular markers. Three questions were addressed: First, does sufficient genetic variation exist to discriminate between pure *O. wolfii* and *O. glazioviana* populations? Second, can hybrid populations be identified using these molecular markers? Third, what is the frequency of hybrid individuals in natural populations of *O. wolfii*? Ultimately, these genetic findings provide greater insight and guidelines for management plans and conservation objectives.

Methods

O. wolfii is a complex heterozygote (Wasmund and Stubbe 1986), or complex hybrid (Bussell et al. 2002), where a diploid individual contains not two copies of a single genome, but a single copy of two distinct genomes. Multiple reciprocal translocations across the genome have produced a single linkage group consisting of both sets of chromosomes at meiosis. As a result, the 14 chromosomes in a diploid individual form a single ring instead of seven bivalents. This phenomenon persists through self-pollination coupled with balanced lethals, with gametophytic and sporophytic lethals persisting in the heterozygous state. Although self-fertilization may produce embryos homozygous for either genome, only heterozygous embryos survive to produce viable seed, since those embryos homozygous for one genome will also be homozygous for either the sporophytic or gametophytic lethal allele. As a result, alleles are not independently assorted, and populations are not randomly mating. This mechanism explains the lack of genotypic diversity and recombination observed in the data set (see Results), and prevents the use of statistical analyses typical of co-dominant genetic data (e.g., admixture analyses or population assignment tests).

Samples were collected from populations at 22 sites whose taxonomy was determined by morphological traits (Tables 1 and 3, Fig. 1). Typical *O. wolfii* plants produce small (<5 cm) pale-yellow corollas having petals that do not overlap, with sepals covered in dense long-spreading pubescence, both villous and glandular pubescence occur on fruits, and plants have a reddish upper stem (Imper 1997). By contrast, typical *O. glazioviana* plants produce larger (>8 cm) bright yellow flowers having substantial overlap in the petals, minimal pubescence on either sepals or fruit, green upper stems, and more wrinkled and lighter green foliage than observed on *O. wolfii* (Imper 1997). Field observations identified four populations as *O. glazioviana* (nos. 1 to 4; the populations of the garden escape most proximate to *O. wolfii*), 14 populations as *O. wolfii* (nos. 6 to 19), and three populations as intermediates or putative hybrids (nos. 20 to 22). Field observations could not distinguish between *O. glazioviana* and *O. elata*, a common congener at one site (no. 5), and one population appeared to be *O. wolfii*, but occurred in a novel location (no. 15). A single leaf was collected from between four and 25 individuals in each population for subsequent genetic analyses.

Tissue was prepared for isozyme analysis following the liquid nitrogen procedure using Gottleib (1981) extraction buffer, as described in NFGEL Standard Operating Procedures (USDA Forest Service 2003). Samples were frozen at −70°C until electrophoresis.

Electrophoresis took place on three buffer systems (adapted from Wendel and Weeden 1989): a tris-citric acid gel buffer (pH 8.3) with a lithium hydroxide-boric acid tray buffer (pH 8.3; LB), a tris-citric acid gel buffer

TABLE 1. POPULATION NUMBER, NAME, LOCATION (LATITUDE, LONGITUDE), AND SPECIES COMPOSITION OF 22 SITES SAMPLED FOR THIS STUDY. Species composition was determined by field observations and genetic analysis, and is indicated by: WO = *O. wolfii*, GL = *O. glazioviana*, HY = intermediate morphology potentially due to hybridization, and UN = unknown taxonomy.

Number	Name	Location	Species
1	Charleston, Coos Co., OR	43.3397N, 124.3308W	GL
2	Crescent City, Del Norte Co., CA	41.7486N, 124.2022W	GL
3	Manila, Humboldt Co., CA	40.8483N, 124.1650W	GL
4	Trinidad, Humboldt Co., CA	41.0353N, 124.1058W	GL
5	Junction City, Trinity Co., CA	40.7378N, 123.0575W	UN
6	Port Orford City Park, Curry Co., OR	42.8320N, 124.5020W	UN
7	Houda Point, Humboldt Co., CA	41.0359N, 124.1187W	WO
8	Port Orford Beach, Curry Co., OR	42.7318N, 124.4825W	UN
9	Port Orford Bridge, Curry Co., OR	42.7318N, 124.4825W	UN
10	Luffenholtz, Humboldt Co., CA	41.0353N, 124.1247W	WO
11	Pistol River, Curry Co., OR	42.2717N, 124.4051W	WO
12	Point St. George, Del Norte Co., CA	41.7778N, 124.2405W	WO
13	Devil's Gate, Humboldt Co., CA	40.4055N, 124.3914W	UN
14	Davis Creek, Humboldt Co., CA	40.3765N, 124.3725W	WO
15	McKerricher State Park, Mendocino Co., CA	39.5146N, 123.7769W	UN
16	Freshwater Spit, Humboldt Co., CA	41.2667N, 124.1058W	WO
17	Crescent Beach, Del Norte Co., CA	41.7194N, 124.1447W	WO
18	False Klamath Cove, Del Norte Co., CA	41.6027N, 124.1064W	WO
19	Crescent Overlook, Del Norte Co., CA	41.7048N, 124.1447W	WO
20	Klamath, Del Norte Co., CA	41.5151N, 124.0298W	HY
21	Lucky Bear Casino, Del Norte Co., CA	41.9529N, 124.2022W	HY
22	Fruit Station, Curry Co., OR	41.9984N, 124.2124W	HY

(pH 8.8) with a sodium hydroxide-boric acid tray buffer (pH 8.0; SB), and a citric acid-N-(3-aminopropyl)-morpholine gel and tray buffer (pH 8.0; MC8). A total of 15 loci were examined. Four loci were resolved on the LB system: phosphoglucose isomerase (PGI2), phosphoglucomutase (PGM1), and two loci in leucine aminopeptidase (LAP1 and LAP2). Four loci were also resolved on the SB system: aspartate aminotransferase (AAT1), superoxide dismutase (SOD1), triosephosphate isomerase (TPI1), and uridine diphosphoglucose pyrophosphorylase (UGPP1). Seven loci were resolved on the MC8 system: two loci in esterase (EST1 and EST2), fluorescent esterase (FEST1), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), and two loci in 6-phosphogluconate dehydrogenase (6PGD1 and 6PGD2). All stain recipes were adapted from Conkle et al. (1982). Banding patterns were consistent with published protein structure (Crawford 1989).

As a consequence of complex hybridity in *O. wolfii*, the isozyme data resolved in this study violate two assumptions common to most statistical analyses designed for genetic data: independent assortment of loci and random mating. Multivariate analyses can often be used to analyze co-dominant genetic data, but these analyses require that errors (or residuals) are normally distributed. This assumption is generally satisfied under independent assortment and random mating, and experience shows that even binary-scored markers can fit the assumption. But, portions of the *Oenothera* isozyme data violate these assumptions. Given the unique nature of the genetic system of *O. wolfii*, and the lack of statistical procedures available to account for complex hybridity as a mode of inheritance, an *ad hoc* approach involving two statistical analyses was employed to determine if hybridization occurs between *O. wolfii* and *O. glaziovianna*: multivariate analyses over populations and individuals, and a maximum likelihood analysis over populations. While acknowledging that neither approach is statistically ideal, we contend that given the unique nature of the genome of *Oenothera* which may bias results of a single statistical test, combining statistical analyses with careful examination of the electrophoretic patterns provides an informative approach to describe the genetic similarities and potential for hybridization between these species.

For the multivariate analysis, we scored each allele as 1.0, 0.5, or 0.0 for homozygous, heterozygous, or homozygous for another allele, respectively (Westfall and Conkle 1992). These scored data were submitted to a canonical discriminant analysis. We first ran the analysis on populations, without respect to species classification to determine if populations grouped by species identity. We then contrasted these results with a classification based on the *apriori* groupings. Based on the canonical discriminant plot, each population was classified as either "pure" (that is, a parental species) or "unknown" (that is, either putative hybrid or unknown taxonomy)

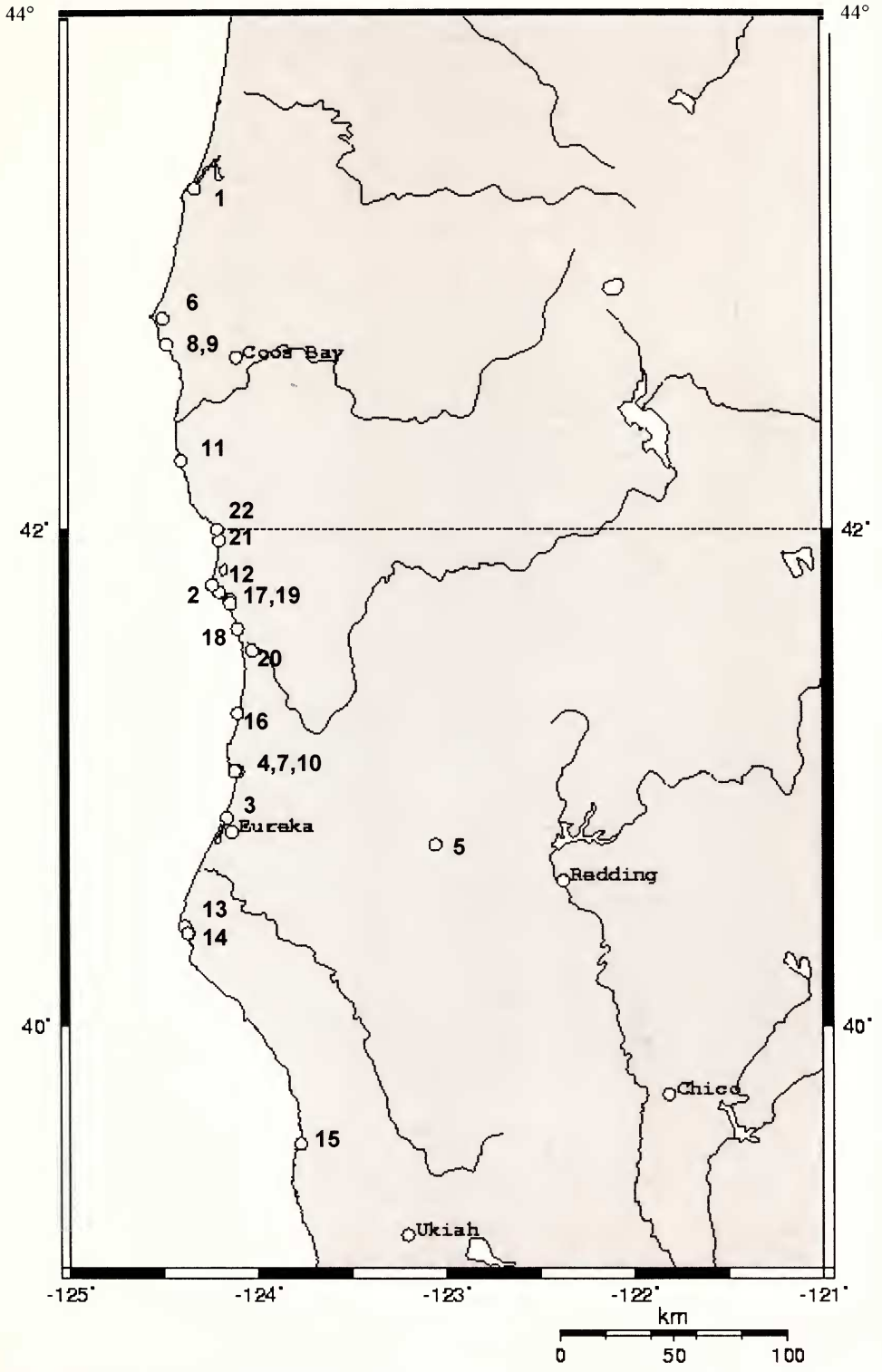

FIG. 1. Location of 22 populations sampled for genetic analysis. Numbers correspond to populations in Table 1.

TABLE 2. ISOZYME DIVERSITY SUMMARY STATISTICS FOR 22 POPULATIONS DESCRIBED IN TABLE 1. MEANS OVER SPECIES INCLUDE ONLY "PURE" POPULATIONS. N = number of samples, P = percent polymorphic loci, A = mean alleles per locus, A_P = mean alleles per polymorphic locus, H_o = observed heterozygosity, F = fixation index. Variance reported in parentheses.

Population	N	P	A	A_P	H_o	F
Mean over species:						
O. wolfii	137	13.33	1.200 (0.293)	2.500	0.021 (0.005)	−0.571
O. glazioviana	61	6.67	1.067 (0.062)	2.000	0.067 (0.062)	−1.000
1	25	0.067	1.067	2.000	0.067	−1.000
2	17	0.067	1.067	2.000	0.067	−1.000
3	11	0.067	1.067	2.000	0.067	−1.000
4	8	0.067	1.067	2.000	0.067	−1.000
5	21	0.133	1.133	2.000	0.133	−1.000
6	6	0.133	1.133	2.000	0.078	−0.750
7	12	0.067	1.067	2.000	0.011	−0.048
8	9	0.067	1.067	2.000	0.067	−1.000
9	9	0.067	1.067	2.000	0.067	−1.000
10	13	0.067	1.067	2.000	0.015	−0.091
11	25	0.000	1.000	n.a.	0.000	0.000
12	19	0.067	1.067	2.000	0.018	−0.125
13	10	0.200	1.200	2.000	0.073	0.214
14	9	0.133	1.133	2.000	0.067	−0.385
15	10	0.000	1.000	n.a.	0.000	0.000
16	25	0.000	1.000	n.a.	0.000	0.000
17	5	0.000	1.000	n.a.	0.000	0.000
18	25	0.067	1.067	2.000	0.067	−1.000
19	4	0.000	1.000	n.a.	0.000	0.000
20	10	0.067	1.067	2.000	0.067	−1.000
21	10	0.067	1.067	2.000	0.067	−1.000
22	5	0.067	1.067	2.000	0.067	−1.000

(Table 1). The allele frequencies observed over all pure populations of each species were then used as the basis to classify each unknown population using the methods described above. These analyses were done in JMP (SAS Institute, Inc, 2004). This software's canonical discriminant analysis is based on Bayesian probabilities, whereby, in well-differentiated species, individuals of one species will have a probability of 1.0, those of the other species, 0.0, and those of hybrid or backcross types will have probabilities between 1.0 and 0.0.

The second analysis estimates the frequency of six genealogical classes (each parental class, first- and second-generation hybrids, and first generation backcross to each parent species) in each population based on the maximum likelihood estimates of the multilocus genotypes observed in a population arising from the allele frequencies observed in each of the pure parental species (Nason et al. 2002). While this method assumes both independent assortment of alleles and random mating within each population, assumptions that are violated here, it includes all loci in the data set in the maximum likelihood estimations, without requiring unique alleles in each parent species (Nason et al. 2002).

Finally, the conclusions from each statistical analysis were considered in the context of the alleles observed in each genotype in the nine unknown populations, with particular attention given to those loci displaying alleles unique to at least one parental species.

RESULTS

Six of the 15 loci examined were polymorphic: 6PGD2, AAT1, UGPP1, FEST1, EST1, and EST2 (Appendix A). Four loci displayed variation within or among populations of the pure species, and two loci contained variation in unknown populations not observed in either pure species. Low levels of genetic variation were observed over all populations surveyed (Table 2). Based on the mean over species, *O. wolfii* contained higher levels of polymorphism (percent polymorphic loci, P = 13.3), alleles per locus (A = 1.20), and alleles per polymorphic locus (A_P = 2.50), but lower levels of heterozygosity (observed proportion of heterozygotes, H_o = 0.021) than *O. glazioviana* (P = 6.67, A = 1.07, A_P = 2.00, H_o = 0.067). Many populations displayed an excess of heterozygotes, as indicated by fixation indices, which is consistent with complex hybridity (Table 2).

Multivariate analyses over populations indicated sufficient genetic differentiation exists to distinguish between *O. wolfii* and *O. glazioviana* (Fig. 2). In general, populations grouped by species identity as defined in field observations, with the majority of populations being separated

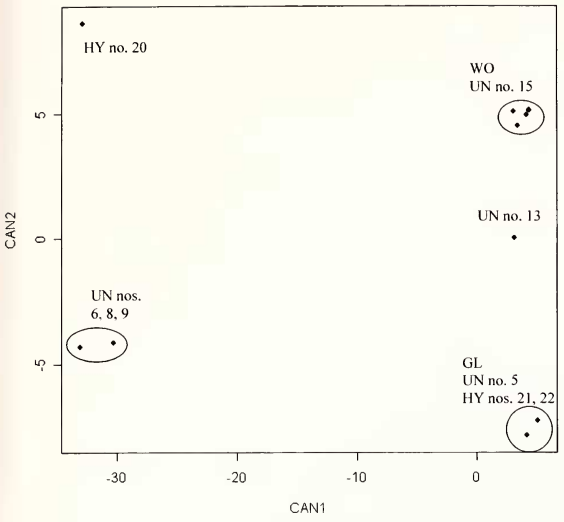

FIG. 2. Distribution of populations along the first two canonical variables produced by a discriminate coordinate analysis. Populations classifications and numbers correspond to Table 1. Can1 = first canonical variable, Can2 = second canonical variable.

by species identification along the second canonical axis (Fig. 2). However, the first two canonical coefficients revealed greater genetic differentiation than predicted among populations classified as *O. wolfii* from morphological characteristics. In particular, three populations from Oregon, nos. 6, 8, and 9, were genetically distinct from the other populations considered pure *O. wolfii* (Fig. 2). Additionally, population 13 was intermediate to the two parental species (Fig. 2). As a result of these observations, these four outlying populations were classified as unknown taxonomy for the remaining statistical tests, reducing the number of *O. wolfii* populations to those listed in Table 1.

No evidence of hybridization (or admixture) was found using the Bayesian classification analysis of individuals. Bayesian tests classified all individuals as either pure *O. wolfii* or pure *O. glazioviana* (Table 3); no intermediate probabilities were observed. Samples from three populations identified as pure *O. wolfii* based on morphological observations were classified as *O. glazioviana* (nos. 6, 8, and 9). Of the three remaining populations of unknown taxonomy, all individuals from one population were classified as *O. glazioviana* (no. 5), all from another as *O. wolfii* (no. 15) and the final population contained a mixture of individuals classified as both pure species (no. 13). Of the three populations classified as hybrid based on morphological observations, genetic analyses classified samples from one as *O. wolfii* (no. 20), and those from the other two as *O. glazioviana* (nos. 21 and 22).

Genealogical class frequency estimates were also inconsistent with morphological predictions (Table 3). Unlike the Bayesian classifications, however, some genotypes were identified as consistent with hybrid origin. Of the populations identified as pure *O. wolfii* a priori, three were classified as hybrids (nos. 6, 8, and 9), and a fourth (no. 13) was classified as a mixture of *O. wolfii* and hybrids. Consistent with the Bayesian classifications, two of the putative hybrid populations were classified as *O. glazioviana* (nos. 21 and 22), although the third was classified as backcross to *O. wolfii* (no. 20).

DISCUSSION

Does sufficient genetic variation exist to discriminate between species?

In genetic studies of hybridization, the genetic variation in each species is often defined by identifying "pure" populations from morphological observations and assaying each for genetic markers. Alternatively, multivariate analyses such as the canonical discriminant analysis described above, can identify genetically similar or distinct populations without a priori classification. In this study, the canonical discriminant

TABLE 3. CLASSIFICATION OF NINE POPULATIONS OF UNKNOWN OR HYBRID ORIGIN BASED ON 6 VARIABLE ISOZYME LOCI. See text for details of the Bayesian classifications and genealogical class freuqencies. Isozyme phenotypes are classified by the presence of alleles found to be unique to either parental species.

Population	Field Observations	Bayesian classification	Genealogical class frequency	Isozyme phenotype
5	Unknown	*O. glazioviana*	Backcross to *O. glazioviana*	Neither species or Hybrid
6	*O. wolfii*	*O. glazioviana*	Hybrid	Hybrid
8	*O. wolfii*	*O. glazioviana*	Hybrid	Hybrid
9	*O. wolfii*	*O. glazioviana*	Hybrid	Hybrid
13	*O. wolfii*	Mix of pure parental individuals	Mix of *O. wolfii* and Hybrid	Mix of *O. wolfii* and Hybrid
15	*O. wolfii*	*O. wolfii*	*O. wolfii*	*O. wolfii*
20	Hybrid	*O. wolfii*	Backcross to *O. wolfii*	Hybrid
21	Hybrid	*O. glazioviana*	*O. glazioviana*	*O. glazioviana*
22	Hybrid	*O. glazioviana*	*O. glazioviana*	*O. glazioviana*

analysis indicated that four populations which were identified as *O. wolfii* in field observations were genetically distinct from the other *O. wolfii* populations (Table 3, Fig. 2). Given the striking genetic differences between these populations, the four outliers were treated as "unknown" taxonomy for the remaining data analyses and interpretation.

The multivariate analysis also indicates that sufficient genetic differentiation exists between the nine *O. wolfii* populations and four *O. glazioviana* populations to discriminate between the parental species (Fig. 2). Although isozyme markers revealed low levels of variation in *O. wolfii* and *O. glazioviana* (Table 2), greater variation was observed in *O. wolfii* (0–20% polymorphic loci) than *O. glazioviana* (6.7% polymorphic loci), and all samples from "pure" *O. glazioviana* populations (nos. 1–4) shared a common genotype: heterozygous at AAT1, and monomorphic at all other loci. *O. wolfii* contained a greater number of alleles per locus (1.20 compared to 1.07 in *O. glazioviana*), and displayed greater levels of fixation (−0.57 compared to −1.00 in *O. glazioviana*).

However, had four populations initially considered *O. wolfii* (nos. 6, 8, 9, and 13) been included in the description of the parental species, the genetic differentiation would have been much less pronounced. Specifically, analyzing these populations as *O. wolfii* would affect the distribution of alleles at locus 6PGD2, making allele 6PGD2-2 no long unique to *O. glazioviana*, but shared between the species. Allele AAT-1 would remain unique to *O. glazioviana*, however. This difference would have likely reduced but not removed the ability of the multivariate and genealogical class frequency analyses to distinguish between pure and hybrid individuals.

These analyses are complicated, however, by the occurrence of alleles in several populations that are not observed in either pure species (Appendix A). There are three possible explanations for these observations. First, these alleles may be present in other populations of either or both parental species that were not sampled for this study. Second, considering populations 6, 8, and 9 as *O. wolfii* as per field observations would make alleles EST1-2, EST2-2, and FEST1-2 unique to *O. wolfii*. As population 20 has consistently been considered of putative hybrid origin, such a change in classification of other populations would not explain the origin of allele FEST1-3. Third, the model we are testing, that all populations are either pure *O. wolfii*, pure *O. glazioviana*, or an admixture of the two, may not explain the genetic structure observed. Past hybridization and introgression between *O. wolfii* and a third, unidentified species (possibly *O. elata*) may explain the high frequency of alternate alleles observed in some test populations. As no data were collected from other *Oenethera* species, we cannot test this alternate model.

Analyzing genetic data from these species and conclusively identifying hybrid individuals is further complicated by the recombination system displayed by *O. wolfii*. As a complex hybrid, putative diploid individuals contain not two copies of a single genome, but one copy each of two distinct genomes. Wasmund and Stubbe (1986) showed that *O. wolfii* maintains this heterozygosity through self-fertilization coupled with balanced lethals. This recombination system causes species to be functional apomicts, typically displaying little genetic variation and heterozygosity (Russell and Levin 1988). The low levels of allelic diversity and near lack of genotypic diversity observed in *O. wolfii* are consistent with these expectations. Although sufficient genetic variation exists to allow differentiation of pure species and identification of hybrid individuals, the lack of recombination and independent assortment at meiosis means that these data violate the assumptions common to most statistical analyses. Thus, standard statistical methods of identifying and monitoring hybrid swarms may not be applicable to *O. wolfii*. In order to appropriately interpret genetic data without loosing information due to violations of model assumptions, comparing the results of multiple statistical analyses coupled with phenotypic descriptions of the multilocus genotypes provides insight into the origin of unknown or putative hybrid populations.

As a final caveat, interpretation of this data set, as well as its application in future studies, must be considered in the context of the small sample sizes at some populations and the small number of pure *O. glazioviana* populations sampled. Analysis of additional "pure" populations of *O. glazioviana* may identify additional unique alleles or reveal alleles thought to be unique to *O. wolfii* to be shared by the two species. Either observation could change the classification of unknown samples and the conclusions herein. Ultimately, this data set represents a fraction of the *Oenothera* genome, and may not completely represent the levels of variation or hybridization in these species.

Can hybrid populations be identified using isozymes?

The genetic differences observed between the nine populations of *O. wolfii* and the four populations of *O. glazioviana* are sufficient to allow identification of hybrid populations. Results of the two statistical analyses are inconsistent, but indicate that hybridization may occur at a rate greater than that expected from morphological observations. The multivariate classification of the six unknown and three putative hybrid

populations identified each collection as either parental species or a mixture of the two (Table 3). The genealogical class frequency estimates, by contrast, only identified three populations as either parental species, and the remaining populations as some hybrid origin (Table 3). This lack of consensus between analyses may be a consequence of the violations of the statistical assumptions these data present. Three general conclusions can be made when the field observations and statistical analyses are considered together. First, populations 6, 8, 9, 13, and 20 are distinct from either parental species. Second, populations 5, 21, and 22 more closely resemble *O. glazioviana* than *O. wolfii*. Third, population 15 is consistent with being *O. wolfii* both morphologically and genetically.

What is the frequency of hybrid individuals in natural populations?

Although population-level analyses to detect hybridization produced inconsistent results, careful consideration of the multilocus genotypes demonstrates that plants from six populations display alleles unique to both parent species, and are thus consistent with hybrid origin (Appendix B). Samples from four populations considered *O. wolfii* from field observations (nos. 6, 8, 9, and 13) contained one allele unique to *O. glazioviana* (6PGD2-2) as well as one unique to *O. wolfii* (UGPP1-2). Had these populations been considered pure *O. wolfii* for the classification tests, and allele 6PGD2-2 would consequently be shared between the parental species. However, populations 6, 8, and 9 also displayed three alleles not observed in either parental species, EST1-2, EST2-2, and FEST1-2. These alleles may be unique to either parental species but not detected in the pure populations, or it may be the result of introgression with another *Oenothera* species (e.g. *O. elata*). As no other species was included in this study, no conclusions can be made regarding the origin of these alleles from these data.

The genotype observed in population 5 is consistent with hybrid origin irrespective of the classification of populations 6, 8, and 9, as it contains the alternate allele unique to *O. glazioviana*, AAT1-2, as well as the allele unique to *O. wolfii*, UGPP1-2. Similarly, the genotype observed in population 20 is also consistent with a hybrid origin, containing the *O. glazioviana* allele AAT1-2 as well as the *O. wolfii* allele 6PGD2-1. However, the genotype in population 20 also contains two alleles observed in populations 6, 8, and 9 (EST1-2 and EST2-2), as well as an allele unique to its population (FEST1-3). Again, given the absence of these alleles in either parental species and the lack of other *Oenothera* species in this study, the origin of these alleles cannot be determined.

Despite violations of assumptions in each statistical analysis, this genetic study reveals evidence of hybridization between the rare endemic *O. wolfii* and the garden escape *O. glazioviana*. A number of genotypes contain alleles found to be unique to each pure species (Appendix B), an observation most easily explained as evidence of hybrid origin. These results indicate hybridization may occur at a greater rate than expected based on morphological observations alone. Although the genetic structure of population 20, a putative hybrid population found to contain an intermediate genotype, demonstrates that not all hybridization events will lead to the genetic swamping of the rare species, timely removal of *O. glazioviana* plants from sympatric sites may be warranted to prevent further loss of the endemic genotype.

While the isozyme loci used here provide alleles unique to each parent species, and thus the ability to identify hybrid individuals, the direction of hybridization and introgression cannot be assessed due to their bi-parental inheritance. Assaying these species for variation at maternally-inherited markers (e.g., chloroplast haplotypes), and combining data from those markers with data from isozyme or other nuclear markers may provide the power to determine which species is serving as the seed donor in each hybridization, and thus determine if *O. wolfii* flowers are being swamped by *O. glazioviana* pollen. In addition, including *O. elata* in future studies would be prudent given the high rates of hybridization between many members of this genus.

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APPENDIX A. ALLELE FREQUENCIES FOR THE SIX VARIABLE LSOZYME LOCI OBSERVED IN THE 22 POPULATIONS DESCRIBED IN TABLE 1.

Locus/Allele	6PGD2		AAT1		EST1		EST2				FEST1			UGPP1	
Population	1	2	1	2	1	2	1	2	3	4	1	2	3	1	2
Mean over species:															
O. wolfii	1.000		1.000		1.000		0.975		0.018	0.007	1.000			0.858	0.142
O. glazioviana		1.000	0.500	0.500	1.000		1.000				1.000			1.000	
1		1.000	0.500	0.500	1.000		1.000				1.000			1.000	
2		1.000	0.500	0.500	1.000		1.000				1.000			1.000	
3		1.000	0.500	0.500	1.000		1.000				1.000			1.000	
4		1.000	0.500	0.500	1.000		1.000				1.000			1.000	
5		1.000	0.500	0.500	1.000		1.000				1.000			0.500	0.500
6		1.000	1.000		0.083	0.917		1.000				1.000		0.500	0.500
7	1.000		1.000		1.000		0.9167		0.083		1.000			1.000	
8		1.000	1.000			1.000		1.000				1.000		0.500	0.500
9		1.000	1.000			1.000		1.000				1.000		0.500	0.500
10	1.000		1.000		1.000		0.885		0.115		1.000			1.000	
11	1.000		1.000		1.000		1.000				1.000			1.000	
12	1.000		1.000		1.000		1.000				1.000			0.868	0.132
13	0.550	0.450	1.000		1.000		0.800			0.200	1.000			0.500	0.500
14	1.000		1.000		1.000		0.889			0.111	1.000			0.500	0.500
15	1.000		1.000		1.000		1.000				1.000			1.000	
16	1.000		1.000		1.000		1.000				1.000			1.000	
17	1.000		1.000		1.000		1.000				1.000			1.000	
18	1.000		1.000		1.000		1.000				1.000			0.500	0.500
19	1.000		1.000		1.000		1.000				1.000			1.000	
20	1.000		0.500	0.500		1.000		1.000					1.000	1.000	
21		1.000	0.500	0.500	1.000		1.000				1.000			1.000	
22		1.000	0.500	0.500	1.000		1.000				1.000			1.000	

Appendix B. Genotypes at Six Variable Isozyme loci Observed in the 22 Populations Described in Table 1. Genotype Code identifies each unique genotype observed in the study. * Genotype contains alleles unique to both parent species.

Population	Genotype Code	6PGD2	AAT1	EST1	EST2	FEST1	UGPP1
1	A	22	12	11	11	11	11
2	A	22	12	11	11	11	11
3	A	22	12	11	11	11	11
4	A	22	12	11	11	11	11
5	B*	22	12	11	11	11	12
6	C*	22	11	22	22	22	12
	D*	22	11	12	22	22	12
7	I	11	11	11	11	11	11
	J	11	11	11	13	11	11
8	C*	22	11	22	22	22	12
9	C*	22	11	22	22	22	12
10	I	11	11	11	11	11	11
	J	11	11	11	13	11	11
11	I	11	11	11	11	11	11
12	I	11	11	11	11	11	11
	K	11	11	11	11	11	12
13	K	11	11	11	11	11	12
	G*	12	11	11	11	11	12
	E*	22	11	11	11	11	12
	F*	22	11	11	44	11	12
14	K	11	11	11	11	11	12
	L	11	11	11	44	11	12
15	I	11	11	11	11	11	11
16	I	11	11	11	11	11	11
17	I	11	11	11	11	11	11
18	K	11	11	11	11	11	12
19	I	11	11	11	11	11	11
20	H*	11	12	22	22	33	11
21	A	22	12	11	11	11	11
22	A	22	12	11	11	11	11